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(54) Title: DETERMINATION OF PREGNANCY STATUS

(57) Abstract: A method and kit for determining whether an animal is not pregnant, or is pregnant following a breeding. The level of expression of a pregnancy induced protein is determined in an animal for which pregnancy status information is desired and the level is compared to that of the level in animals that are not pregnant.

WO 02/103352 A1

## Determination of Pregnancy Status

### Field of the Invention

The invention relates to methods for determining the pregnancy status of animals. Particularly, the invention relates to methods for determining the pregnancy status of non-ungulates, ungulates, and ruminant animals.

### Background of the Invention

In the rearing of livestock, it is very important to accurately determine the pregnancy status of bred animals. In particular, it is the accurate and early identification of failed pregnancy of an animal that has been bred that is economically important. Presently, once an animal is bred, for example a cow, pregnancy status is determined by such methods as palpation, which does not provide an accurate determination of pregnancy status until after 30 days following breeding. Because cattle have an estrous cycle of about 21 days, this means that with presently available methods at least one opportunity for breeding an animal that fails to conceive, the estrus period immediately following the failed breeding, will be missed.

This has important economic consequences for the cattle breeding industry, especially for the dairy industry. Efficient milk production farming requires that cows be successfully bred to become pregnant 80-100 days after

calving. Dairy cows, however, have a low fertility rate with artificial insemination, requiring, on average, 2.5 to 3 inseminations per conception. Therefore, a significant need exists for a method by which a dairy farmer may accurately  
5 determine that an animal is not pregnant without missing an opportunity to re-breed the animal at the next estrus period following an unsuccessful breeding.

Sasser, U.S. Patent No. 4,705,748, incorporated herein by reference, discloses a method for determining pregnancy by  
10 detecting a protein produced by a conceptus. By this method, cattle were determined to be pregnant as early as day 27 following breeding. Sasser does not disclose the diagnosis of pregnancy prior to the time when a subsequent estrus period will have commenced in non-pregnant cattle and does not  
15 disclose an early determination of non-pregnancy.

Maternal recognition of pregnancy in ungulates involves local and systemic gene regulation by the conceptus that results in reduced or altered production of the luteolytic signal, prostaglandin F2 $\alpha$  (PGF2 $\alpha$ ); Yankey et al., Expression  
20 of the antiviral protein Mx in peripheral blood mononuclear cells of pregnant and bred, non-pregnant ewes. Journal of Endocrinology 170, R7-R11 (2001); Bazer et al., Regulation of endometrial responsiveness to estrogen and progesterone by pregnancy recognition signals during the peri-implantation  
25 period. In Molecular and Cellular Aspects of Peri-implantation Processes, pp 27-47. Ed S.K. Dey. Springer-Verlag, New York,

Inc. (1995)). This is in contrast to pregnancy recognition in primates, which involves a direct luteotrophic effect on the corpus luteum (CL) by conceptus-produced chorionic gonadotropin (Bazer et al. 1995). The signal for maternal  
5 recognition in ungulates is the secretion by the conceptus of interferon-tau (IFN $\tau$ ) during the second and third week of pregnancy (Bazer et al., 1995; Godkin et al., J. Reprod. Fert. 65:141-150(1982)). IFN $\tau$  prevents increases in endometrial estrogen and oxytocin receptors, to abrogate oxytocin-induced  
10 luteolytic pulses of PGF2 $\alpha$ , and maintains CL function (Spencer et al., Endocrinology 136:4932-4944 (1995)).

IFN $\tau$  is a member of the Type I IFN family, which also includes IFN  $\alpha$ ,  $\beta$ , and  $\omega$  (Samuel, Virology 183:1-11 (1991)), and, more recently, interferon  $\delta$  (Lefevre, F., et al.,  
15 Biochimie 80:779-788 (1998). IFN $\tau$  signaling through the Type I IFN receptor and Janus Kinase (JAK)-signal transducer and activator of transcription (STAT) signal transduction pathway (Stewart et al., Endocrinology 142:98-107 (2001)) induces a number of genes in the ovine uterus including 2',5'  
20 oligoadenylate synthetase (Johnson et al., Biol. Reprod. 64:1392-1399 (2001)),  $\beta$ 2-microglobulin (Vallet et al., J. Endocrinol. 130:R1-4 (1991)), IFN regulatory factor 1 (Spencer et al., 1998), ubiquitin cross-reactive protein (Johnson et al., Biol. Reprod. 62:622-627(2000)), and Mx protein  
25 (Charleston and Stewart, Gene 137:327-331(1993); Ott et al., Biol. Reprod. 59:784-794 (1998)). While the functions of many

of these proteins in the antiviral response are well characterized, their roles during early pregnancy are not.

Mx proteins are monomeric GTPases, which, depending on the species of animal and type of virus, are potent inhibitors of viral replication (Samuel, Virology 183:1-11 (1991)). The sequences of Mx proteins from various species, including sheep, cattle, pigs, and horses, are publicly available through GenBank and have been assigned GenBank Accession numbers X66093, U88329, M65087, and U55216, respectively.

Although the antiviral effects of Mx are generally directed against negative-stranded RNA viruses (e.g. orthomyxovirus), their expression is induced in all cells that possess Type I IFN receptors and has been used to distinguish between bacterial and viral infection (Haller et al., Rev. Sci. Tech. 17:220-230 (1998)). Recently Mx mRNA and protein were shown to be elevated from epithelium (by day 13) to myometrium (by day 15) within the uterine wall in pregnant ewes and levels remained elevated through day 25 (Ott et al., Biol. Reprod. 59:784-794 (1998)). In addition, Mx mRNA levels were elevated in the corpus luteum in response to injections of roIFN $\gamma$  into the uterine lumen (Spencer et al., Biol Reprod 61:464-470 (1999)).

These results indicated that IFN $\gamma$  was either: 1) acting directly on all uterine cell types (i.e., epithelial, stromal and myometrial) and on the CL; or 2) inducing substances (cytokines) that have paracrine/endocrine effects on uterine

cells and other organs including the ovaries; or 3) affecting components of the uterine mucosal and circulating immune systems which then affect the various uterine cells and CL.

It is impractical, however, to measure the level of Mx protein in uterine tissue as a test for evaluating pregnancy status. Besides being an invasive and time and labor intensive process, the disruption of uterine tissues necessary to determine the uterine levels of Mx would tend to have a deleterious effect on a pregnancy.

A significant need exists for a reliable, reproducible, and non-invasive method for determining pregnancy or lack of pregnancy in domestic livestock.

#### Summary of the Invention

It has been discovered that the expression of the genes encoding for several proteins, herein referred to as "pregnancy-induced proteins", including 2',5' oligoadenylate synthetase,  $\beta$ 2-microglobulin, IFN regulatory factor 1, ubiquitin cross-reactive protein (also known as "interferon stimulated gene factor 17" ("ISG-17")), and Mx protein, increases significantly in certain animals during the first month of pregnancy. It has further been discovered that the increase in the expression of the pregnancy-induced proteins does not occur in animals that are not pregnant.

In many animals, the increase in expression of the pregnancy induced protein is due to the secretion by the

embryo of a hormone, Type I interferon, that is the signal from the embryo to the mother of its existence, referred to as the signal for maternal recognition of pregnancy. Different type I interferons, including Interferon alpha ( $\text{IFN}\alpha$ ),  
5 Interferon beta ( $\text{IFN}\beta$ ), Interferon omega ( $\text{IFN}\omega$ ), Interferon delta ( $\text{IFN}\delta$ ), and Interferon tau ( $\text{IFN}\tau$ ) are secreted by the embryos of different species. For example,  $\text{IFN}\tau$  is secreted as a pregnancy recognition hormone in ruminants and  $\text{IFN}\delta$  is secreted in swine. In other species, such as horses and other  
10 equidae, although the pregnancy-induced protein Mx protein is detectable in the uterus during early pregnancy, to date the secretion by the equine conceptus of a Type I interferon has not been demonstrated. Rather, in equines and other species whose conceptuses do not produce a Type I IFN, it is possible  
15 that the uterus produces Type I interferon in response to the presence of the embryo.

In one embodiment, the invention is a method for determining the pregnancy status of an animal. According to this embodiment of the invention, the level of expression of a  
20 pregnancy induced protein during early pregnancy is determined and compared to the level of the expression of that pregnancy induced protein during the same period in a non-pregnant female animal of the same species. Preferably, the pregnancy induced protein that is determined and compared is a Type I  
25 interferon-induced protein. Most preferably, the pregnancy induced protein is Mx protein or ISG-17.

As used herein, the term "pregnancy-induced protein" refers to a protein that is expressed by a maternal gene and which expression is induced in response to the presence of a pregnancy. A pregnancy-induced protein is distinct from a protein that is produced by the conceptus, unless such protein is also expressed by a maternal gene and this maternal expression is induced in response to the presence of a pregnancy.

As used herein, the term "early pregnancy" refers to that time during or following the period of pregnancy recognition signaling in which the level of a pregnancy induced protein is elevated in a pregnant animal compared to a non-pregnant animal of the same species. Although animals that are bred unsuccessfully, that is do not become pregnant, may or may not undergo a period of pregnancy recognition signaling, the term "early pregnancy" is used herein with regards to non-pregnant animals to refer to the period of time in which there would be an early pregnancy if the breeding had been successful. Typically, the period of early pregnancy, as used in relation to the method of the invention, ends at about the end of the first month following conception.

As used herein the "period of pregnancy recognition signaling" refers to that time during which the embryo secretes a protein or hormone, the secretion of which causes recognition by the mother of the existence of the embryo. Although animals that are bred unsuccessfully, that is do not



become pregnant, may or may not undergo a period of pregnancy recognition signaling, the term is used herein with regards to non-pregnant animals to refer to the period of time in which, had the breeding been successful, biochemical signaling would be occurring between the conceptus and the uterus.

In accordance with the invention, an animal that is pregnant will exhibit a markedly higher level of expression of one or more pregnancy-induced proteins, such as Mx protein or ISG-17, during early pregnancy, such as during the period of pregnancy recognition signaling, than will a non-pregnant animal of the same species. A non-pregnant animal, whether or not the animal has been bred will exhibit about the baseline level of the pregnancy-induced protein expression, including Mx protein, during this period.

In another embodiment, the invention is a kit for determining the reproductive status of an animal of a species in which the conceptus secretes a protein or hormone as a signal for maternal recognition of pregnancy. According to this embodiment of the invention, the kit includes a receptacle for holding a test sample, one or more reagents which when combined with the test sample enable an operator to visually determine the level of one or more pregnancy-induced protein, such as Mx protein or ISG-17, in the test sample, and instructions for determining the level of the protein in the sample. Preferably the kit further contains instructions that

enable the operator to determine the pregnancy status of the animal based on the determined level of protein in the sample.

The invention is further illustrated below with reference to Mx protein. One skilled in the art will understand that the disclosure below is applicable to other pregnancy-induced proteins, such as other Type I interferon-induced proteins such as IFN $\gamma$  induced proteins, including 2',5' oligoadenylate synthetase,  $\beta$ 2-microglobulin, IFN regulatory factor 1, and ubiquitin cross-reactive protein, as well as to the illustrated Mx protein. Therefore, in the following disclosure, at the mention of the term "Mx protein", any other pregnancy-induced protein may be substituted.

#### Brief Description of the Figures

Figure 1 is a Northern Blot analysis of Mx mRNA from PBMC (peripheral blood mononuclear cells) at day 26 following artificial insemination in ewes. Lanes 1-6 represent pregnant ewes and lanes 8-13 represent non-pregnant ewes. Mx mRNA migrated at ~ 2.5 kb.

Figure 2 is a graph showing the results of a slot blot analysis of total cellular RNA isolated from PBMC at day 26 following artificial insemination in ewes. Mx mRNA levels were about 4 fold greater in pregnant versus bred, non-pregnant ewes at D 26 ( $P < 0.01$ ). PSPB (pregnancy-specific protein B) levels ( $\blacklozenge$ ) confirmed pregnancy status and were

correlated with number of lambs born. (CNTS in Fig. 2 refers to photon counts on a chemiluminescent Northern blot.)

Figure 3 is a bar graph showing the expression of Mx mRNA in PBMC from artificially inseminated pregnant and non-  
5 pregnant ewes from Day 0 to Day 30 following the artificial insemination. (CNTS in Fig. 3 refers to photon counts on a chemiluminescent slot blot.)

Figure 4 is a Western Blot analysis of Mx protein expression from PBMC in pregnant and non-pregnant ewes 15 and  
10 18 days following artificial insemination.

#### Detailed Description of the Invention

The present invention is applicable to any animal species that secretes increased levels of one or more pregnancy induced proteins during early pregnancy. Preferably, the  
15 animal species is one that secretes a Type I interferon as a signal for maternal recognition of pregnancy. Most preferably, the animal species is one that secretes the hormone IFN $\gamma$  as a signal for maternal recognition of pregnancy. In these animals, the increased levels of Type I  
20 interferon, such as IFN $\gamma$ , induces an increased expression of Mx protein during the period of pregnancy recognition signaling. According to the invention, the lack of an increase in expression of a pregnancy-induced protein, such as Mx protein, in a suitable animal during early pregnancy,  
25 preferably the period of time which would encompass the period

of pregnancy recognition signaling following a breeding, is a positive indication that the animal is not pregnant.

Conversely, a negative result, that is the presence of an increase in expression of the protein during this period is an indication that the animal is pregnant.

In this application, the lack of an increased expression of the pregnancy induced protein, such as Mx protein, is referred to as a positive result whereas the presence of an increased expression of the protein is referred to as a negative result. This terminology, which might at first appear to be contrary to the usual usage of the terms "positive" and "negative" result, is utilized herein because it is the finding of non-pregnancy, rather than of pregnancy, which is of most concern to a farmer or rancher or other person engaged in animal husbandry. If an animal is determined to be pregnant, no additional work is expended to ensure that she is indeed pregnant, outside of watching her to look for signs that the pregnancy has been terminated. In contrast, if an animal is determined to be not pregnant, then she must be further evaluated for the onset of her next estrus and will be bred again. Therefore, it is the finding of non-pregnancy that provides the impetus for additional labor to be expended upon the animal to ensure that she does indeed become pregnant.

As stated above, the invention is applicable to any female animal belonging to a species that produces increased

levels of a pregnancy-induced protein during early pregnancy, such as an animal of a species in which a Type I interferon such as IFN $\gamma$  is the sole signal or one of more than one signal for maternal recognition of pregnancy. Animals suitable for the method of the invention include ungulates and non-hoofed ruminants. The ungulates may be ruminants, such as cattle, sheep, goats, yak, water buffalo, and bison. Included among the ungulate ruminants suitable for the invention are also non-domesticated ungulates such as antelopes, gazelles, elk, reindeer, moose, bighorn sheep, giraffes, and other members of the cattle, sheep, and goat families. Ruminant non-ungulates suitable for the method of the invention include bactrian and dromedary camels and other camellids, such as llamas, alpacas, and vicunas. Ungulate non-ruminants suitable for the invention include domesticated and non-domesticated swine and horses. Animals suitable for the method of the invention include animals other than ungulates and non-hoofed ruminants. For example, it is conceived that primates such as humans, as well as dogs and cats, are suitable for the method of the invention.

In accordance with the method of the invention, during an appropriate time period following breeding, an animal is tested for the presence, or more precisely for the lack of presence, of an increased expression of a pregnancy-induced protein, exemplified hereafter as Mx protein. The test may be

performed utilizing any cell in which Mx protein is expressed or in any bodily fluid in which Mx protein is found.

When determining whether an animal has or does not have an increase in expression of Mx protein, a comparison is made to the level of expression in animals that are known to be not pregnant. The comparison may be made, for example, by running a side-by-side comparison of a test sample from an animal that has been bred and which the pregnancy status is uncertain.

Alternatively and preferably, the comparison is made by testing a sample from an animal that has been bred and which the pregnancy status is uncertain and comparing the level of Mx protein in the sample to the level of Mx protein known to be present in non-pregnant animals, that is using a historical control.

The invention is illustrated herein with reference to determining the increased expression of Mx protein in peripheral blood mononuclear cells (PBMC). However, any cell in which Mx protein is expressed, including other nucleated cells present in the bloodstream, may be utilized in place of PBMC. Likewise, it is conceived that increased Mx expression in accordance with the invention may be determined by analysis of fluids, such as milk, saliva, urine, or nasal, ocular, or vaginal secretions, or whole blood, plasma, or serum.

Although the period of maternal recognition in most species occurs about the same time, for example at days 12 to 14 following breeding in ewes and days 15 to 18 in cows, the

period of embryonic signaling that results in maternal recognition of pregnancy varies somewhat amongst different species. Accordingly, the actual dates following breeding on which the method of the invention may be effectively practiced will vary. For example, the period of maternal recognition of pregnancy signaling in domesticated sheep typically begins about day 11 following breeding and continues to about day 21. In cattle, this period typically begins about day 13 and continues to about day 35.

When comparing the level of Mx protein expression in a test sample to the baseline, (non-pregnant) level of Mx protein expression, typically a doubling or higher in Mx protein expression over the baseline is a negative test, that is the animal is not determined to be not pregnant. Usually, at peak levels of Mx protein expression during the period of pregnancy recognition signaling, a pregnant animal will have levels of Mx protein expression that are up to four or five times, or higher, that of baseline.

In accordance with the invention, the method may be practiced at any time commencing with the onset of the period of signaling until the time that the level of the pregnancy induced protein is no longer elevated in pregnant animals compared to non-pregnant animals of the same species. It is during this time that pregnant animals have an increased expression of Mx protein compared to non-pregnant animals. Thus, in sheep and cattle, a preferred time period for

comparing the level of Mx protein expression to determine pregnancy status is between 12 and 30 days following breeding. A more preferred time period is between days 12 and 21 following breeding. A most preferred time period between days 15 and 21, and a most preferred time is on day 18 in cattle and day 15 in sheep.

The level of Mx protein expression may be determined by any method that permits this determination to be made. Suitable methods include detecting the Mx protein itself, such as by ELISA test, an assay based on Mx protein function, or a Western blot. Suitable methods also include detecting increased levels of Mx mRNA, such as by Northern blot, slot blot, or PCR. In a preferred embodiment, the level of Mx protein expression is determined by detecting the level of Mx protein present in a sample by a colorimetric assay based, for example, on the binding of an antibody to the Mx protein, similarly to the methods that are used in human home pregnancy diagnostic kits.

The method of the invention is an accurate, reproducible test that predictably determines that an animal is not pregnant, and may likewise be used to determine that an animal is pregnant. With regards to Mx protein in particular, but not necessarily the other pregnancy induced proteins, the only source of false negative results that would erroneously indicate that the animal is pregnant, that is an increased



level of Mx protein expression, is if the animal is suffering from a severe viral infection.

The kit of the invention is preferably based on an enzyme linked assay (ELISA), such as what is known as an

5 "immunometric" or "sandwich" assay. Such an assay involves "sandwiching" a ligand (such as an antigen) with two or more receptor molecules (such as antibodies) which complex with the ligand in a non-interfering manner and at different epitopic sites. Examples of such assays are described in David et al.,  
10 U.S. Pat. No. 4,486,530. Alternatively, the kit may be based on chemiluminescence assays, enhanced luminescence assays, and radioimmunoassays. In a preferred embodiment, the kit includes a package, which package houses a test surface, such as a slide or multiple test wells, that is bound to an  
15 antibody that will bind to an epitope of the protein of interest, such as Mx protein, a container housing a second antibody that will bind to a second epitope of the protein, which second antibody is labeled, a container housing a standard sample having a baseline concentration of the  
20 protein, a reagent that when contacted to the labeled second antibody permits the relative amount of the protein present to be visualized, and instructions for use of the kit to determine whether a test sample contains an amount of Mx protein indicative of pregnancy or non-pregnancy status.

25 The kit of the invention for determining pregnancy status by determining the relative level of a pregnancy induced

protein, such as Mx protein, in a test sample compared to a control may be formulated in many different ways, which ways will be apparent to those skilled in the art upon reading the description herein. It is intended that these various  
5 formulations of the kit of the invention are included in the invention.

The invention is further described in the following illustrative, non-limiting, examples. The examples describe the method of the invention with reference to Mx protein.  
10 However, the method of the invention is applicable to other pregnancy induced proteins.

#### Example 1 Animal Models

Sixty (60) mature, white-faced, ewes from the U.S. Sheep Experiment Station (USSES, Dubois ID) were synchronized and  
15 bred either by transcervical or laparoscopic artificial insemination ("AI"). Laparoscopic AI was performed according to the procedure disclosed in Stellflug et al., J. Anim. Sci. 79:568-573 (2001). The day of artificial insemination was designated Day 0 (D0). At 26 days after AI (D26), blood (10  
20 ml) was collected by jugular venipuncture into EDTA-containing vacutainer tubes (Sherwood Medical, St. Louis MO). PBMC were isolated as described below in Example 2. Pregnancy was determined by assaying serum for pregnancy-specific protein B (PSPB; Biotracking Inc, Moscow ID) and lambing dates and  
25 number of lambs born were recorded.

## Example 2. PBMC isolation

Blood was kept on ice until processed. Samples were centrifuged at 300 x g for 20 min at 4 C. The buffy coat was removed and resuspended in 0.87% Tris-NH<sub>4</sub>CL lysis buffer at a 1 to 5 ratio. Samples were incubated for 5 min at 37°C and centrifuged at 300 x g for 10 min. The supernatant was removed and pellets were washed with 10 ml 1X PBS and centrifuged for 10 min at 300 x g. After removal of supernatant, cell pellets were either frozen at -80°C for protein extraction, or lysed with 2 ml TRIZOL (Life Technologies, Grand Island NY) and stored at -80°C for RNA extraction.

## Example 3. RNA extraction, Northern and slot-blot analysis.

Total cellular RNA was extracted using TRIZOL according to manufacture's instructions. RNA was quantified by A260:280 ratio. To establish size and number of Mx transcripts in PBMC, RNA (5 µg) was electrophoresed in a 1% agarose/0.615 M formaldehyde gel and transferred to a nylon membrane (Nytran, Schleicher & Schuell, Keene NH) by capillary blotting. For quantification of Mx mRNA levels in PBMC, RNA (5 µg) was transferred to a nylon membrane by vacuum filtration (Minifold II, Schleicher & Schuell, Keene NH). Blots were probed with a biotin-labeled ovine Mx anti-sense cRNA probe (Ott et al., Biol. Reprod. 59:784-794 (1998)) using the North2South

Hybridization kit (Pierce, Rockford IL) and chemiluminescent signal was quantified using a Bio-Rad Fluor-S MultiImager system and Quantity One software (Bio-Rad, Hercules CA). Slot-blots were stripped and re-probed with an ovine 18s rRNA cRNA probe to correct for variations in RNA loading.

Northern blot analysis, as shown in Fig. 1, detected a single, approximately 2.5 kD, band in PBMC isolated from pregnant and bred, non-pregnant ewes, which agrees with the known size of the ovine uterine Mx cDNA (Charleston and Stewart, Gene 137:327-331(1993); Ott et al., Biol. Reprod. 59:784-794 (1998)).

Slot blot analysis, as shown in Fig. 2, of total cellular RNA isolated from PBMC collected at D26 post-AI showed a four-fold increase in Mx mRNA levels in pregnant versus bred, non-pregnant (n=26) ewes ( $P<0.01$ ). In addition, ewes carrying multiples (triplets or quads; n=10) had higher Mx mRNA levels than those carrying singles (n=10) or twins (n=9;  $P<0.05$ ). Results from the PSPB (pregnancy-specific protein B) assay confirmed pregnancy status and, as reported previously, levels of PSPB were correlated with number of lambs born (Willard et al., J. Anim. Sci. 73:960-966 (1995)).

Example 4. Temporal expression of Mx protein mRNA during early pregnancy in sheep

A second study examined the temporal expression of Mx mRNA during early pregnancy in sheep, as shown in Fig. 3.

Thirty four (34) mature Suffolk ewes were synchronized and bred by laparoscopic AI. Blood (20 ml) was collected by jugular venipuncture at D0, and every three days from D9 to D30, and PBMC were isolated. Pregnancy was confirmed by real-time ultrasonography and PSPB assay at D30. Results shown in Fig. 3 are a representative subset of all ewes and depict results from four pregnant and four bred, non-pregnant ewes during the first 30 days following insemination. This allowed analyzing all replicates on a single blot to eliminate problems associated with signal intensity between blots. Results showed Mx mRNA levels increased in pregnant ewes beginning at D15 ( $P < 0.01$ ). Levels peaked at D21 and gradually declined thereafter. At D30, Mx levels in pregnant ewes remained elevated two-fold compared to bred, non-pregnant ewes ( $P < 0.01$ ).

Example 5. Protein isolation and Western blot analysis.

Total cellular protein was extracted using M-PER reagent (Pierce, Rockford IL), according to manufacturers instructions. Protein concentration of samples was quantified by BCA assay (Pierce, Rockford IL) with bovine serum albumen as the standard. Proteins (8  $\mu\text{g}/\text{sample}$ ) from PBMC isolated from pregnant and bred, non-pregnant ewes at D15 and D18 were separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (BA83, Schleicher & Schuell, Keene NH). Following blocking of non-specific binding sites

in 5% non-fat dry milk in Tris-buffered saline and Tween 20 (TBST) for 2 hours at 25°C, membranes were incubated with a 1:1000 dilution of a polyclonal rabbit ovine Mx peptide antiserum (#90618-2; 0.7µg/ml) at 4°C overnight. Goat anti-rabbit IgG (0.8 µg/ml) labeled with horseradish peroxidase was used at a 1:200,000 dilution as secondary antibody.

Chemiluminescent signal was developed using the West Femto Maximum Sensitivity Substrate (Pierce, Rockford IL) and quantified using the Fluor-S MultiImager system and Quantity One software.

As shown in Fig. 4, Mx protein (~75 kDa) was not detected in either D15 or D18 open (non-pregnant) ewes, but was strongly up-regulated in PBMC from pregnant ewes on both days. Two additional bands (~48 and 36 kDa) were detected in PBMC from pregnant ewes.

#### Example 6. Analysis.

Chemiluminescent signal was analyzed using GLM procedures of SAS (Version 8.1, SAS Inc, Gary NC). The model included, where appropriate, status (pregnant versus bred, non-pregnant), ewe nested within status, day (0, 9, 12, 15, 18, 21, 24, 27, and 30) and appropriate interactions. Error terms in the F test were according to the expectation of mean squares for error. Signal for 18s rRNA was run as a covariate in the model to correct for variations in loading. Results

are reported as adjusted Least Squares Means (LSM) and pooled standard errors.

Example 7. Cattle

Thirty three dairy cows were bred by artificial  
5 insemination and their levels of Mx mRNA were determined by  
the method described above in Examples 2 and 3. On day 15,  
levels of Mx mRNA were found to be about the same in cows that  
were later determined to be pregnant and in cows that were  
later determined not to be pregnant. On day 18, levels of Mx  
10 mRNA were found to be have increased markedly in cows later  
determined to be pregnant to about three times the level found  
in cows later determined not to be pregnant.

The results demonstrate a rapid and sustained activation  
of Mx gene expression in response to pregnancy recognition  
15 signaling, and indicate that, in addition to local effects of  
IFN $\gamma$ , there is rapid systemic response in sheep and cattle.  
In addition, Mx expression did not increase in PBMC when  
pregnancy was not established (bred, non-pregnant animals).  
These findings are significant because pregnancy recognition  
20 signaling by IFN $\gamma$  was heretofore considered to result solely  
from local regulation of endometrial gene expression (Stewart  
et al., Endocrinology 142:98-107 (2001); Johnson et al., Biol.  
Reprod. 64:1392-1399 (2001); Vallet et al., J. Endocrinol.  
130:R1-4 (1991); Spencer et al., Biol. Reprod. 58:1154-1162

(1998); Johnson et al., Biol. Reprod. 62:622-627 (2000);  
Charleston and Stewart, Gene 137:327-331(1993); Ott et al.,  
Biol. Reprod. 59:784-794 (1998); Spencer et al., Biol. Reprod.  
61:464-470 (1999)) and suppression of estrogen and oxytocin  
5 receptor expression to abrogate luteolytic pulses of  $\text{PGF}_{2\alpha}$ .  
The methods and kits of the invention therefore provide new  
and economically important methods of non-pregnancy, and  
pregnancy, determinations in livestock.

10 All articles and patents cited in this application are  
incorporated herein by reference.

Further modifications, uses, and applications of the  
invention described herein will be apparent to those skilled  
in the art. It is intended that such modifications be  
encompassed in the following claims.



## Claims:

1. A method for determining pregnancy status in an animal comprising determining the level of expression of a pregnancy-induced protein in the animal during the period of  
5 early pregnancy following breeding of the animal and comparing the level of expression of said protein in the animal to that of a non-pregnant animal of the same species.

2. The method of claim 1 wherein the pregnancy induced protein is a Type I Interferon-induced protein.

10 3. The method of claim 1 wherein the Type I Interferon-induced protein is an Interferon-tau (IFN $\tau$ )-induced protein.

4. The method of claim 1 wherein the animal is a ruminant.

15 5. The method of claim 4 wherein the non-ungulate ruminant is selected from the group consisting of bactrian camel, dromedary camel, llama, alpaca, and vicuna.

6. The method of claim 4 wherein the animal is an ungulate.

7. The method of claim 6 wherein the animal is selected from the group consisting of cattle, sheep, goats, yaks, water buffalos, bisons, antelopes, and deer.

8. The method of claim 7 wherein the animal is a ewe or  
5 a cow.

9. The method of claim 1 wherein the animal is an ungulate.

10. The method of claim 9 wherein the animal is a horse or a swine.

10 11. The method of claim 1 wherein the protein is selected from the group consisting of 2',5' oligoadenylate synthetase,  $\beta$ 2-microglobulin, IFN regulatory factor 1, ubiquitin cross-reactive protein, and Mx protein.

12. The method of claim 11 wherein the protein is Mx  
15 protein.

13. The method of claim 1 wherein the level of expression of the protein is determined by determining the level of mRNA coding for the protein.

14. The method of claim 13 wherein the determination of mRNA is by Northern blot analysis, slot-blot analysis, or polymerase chain reaction.

15. The method of claim 1 wherein the level of  
5 expression of the protein is determined by determining the level of the protein.

16. The method of claim 15 wherein the determination of the level of production of the protein is by evaluating the binding of an antibody to the protein or by an assay based on  
10 a function of the protein.

17. The method of claim 15 wherein the level of production of the protein is detected by a colorimetric assay.

18. The method of claim 1 wherein the level of  
expression of the protein is determined by determining the  
15 expression of the protein in a cell of the animal.

19. The method of claim 18 wherein the cell is a nucleated blood cell.

20. The method of claim 19 wherein the cell is a peripheral blood mononuclear cell.

21. The method of claim 1 wherein the level of expression of the protein is determined by analyzing a bodily fluid of the animal.

22. The method of claim 21 wherein the bodily fluid is selected from the group consisting of milk, saliva, urine, or nasal, ocular, or vaginal secretions.

23. The method of claim 21 wherein the bodily fluid is blood, plasma, or serum.

24. The method of claim 1 wherein the animal is determined to be not pregnant if the level of expression of the protein in the animal during the period of early pregnancy is not elevated compared to the level of expression of the protein in a non-pregnant animal of the same species.

25. The method of claim 1 wherein the animal is determined to be pregnant if the level of expression of protein in the animal during the period of early pregnancy is two times or more the level of expression of the protein in a non-pregnant animal of the same species.

26. The method of claim 25 wherein the animal is determined to be pregnant if the level of expression of the

protein in the animal during the period of early pregnancy is four times or more the level of expression of the protein in a non-pregnant animal of the same species.

27. The method of claim 1 wherein the comparison is by  
5 side-by-side comparison of the level of expression of the protein in the animal and the level of expression in an animal known to be pregnant or not pregnant.

28. The method of claim 1 wherein the comparing is by using an historical control.

10 29. The method of claim 1 which is performed during the period of pregnancy recognition signaling.

30. The method of claim 1 which is performed during the period between 12 and 30 days after breeding of the animal.

15 31. The method of claim 30 which is performed during the period between 12 and 21 days after breeding of the animal.

32. The method of claim 31 which is performed during the period between 18 and 21 days after breeding of the animal.

33. A kit for determining the pregnancy status of an animal, comprising a container for holding a test sample, one

or more reagents which, when combined with the test sample in the container, enable an operator to visually determine the level of a pregnancy-induced protein in the test sample, and instructions for determining the pregnancy status of the animal based upon the level of the protein in the sample.

34. The kit of claim 33 wherein the pregnancy induced protein is an interferon-tau (IFN $\tau$ ) induced protein.

35. The kit of claim 34 wherein the IFN $\tau$  induced protein is Mx protein.

36. The kit of claim 34 wherein the IFN $\tau$  induced protein is ISG-17.

37. A kit for determining the pregnancy status of an animal, comprising a test surface that is bound to an antibody that will bind to an epitope of a pregnancy-induced protein, a container housing a second labeled antibody that will bind to a second epitope of the protein, a container housing a standard sample having a baseline concentration of the protein, a reagent that when contacted to the labeled second antibody permits the relative amount of the protein present to be visualized, and instructions for use of the kit to determine whether a test sample contains an amount of the protein indicative of pregnancy or non-pregnancy status.

38. The kit of claim 37 wherein the pregnancy induced protein is an IFN $\gamma$  induced protein.

39. The kit of claim 38 wherein the IFN $\gamma$  induced protein is Mx protein.

5        40. The kit of claim 38 wherein the IFN $\gamma$  induced protein is ISG-17.

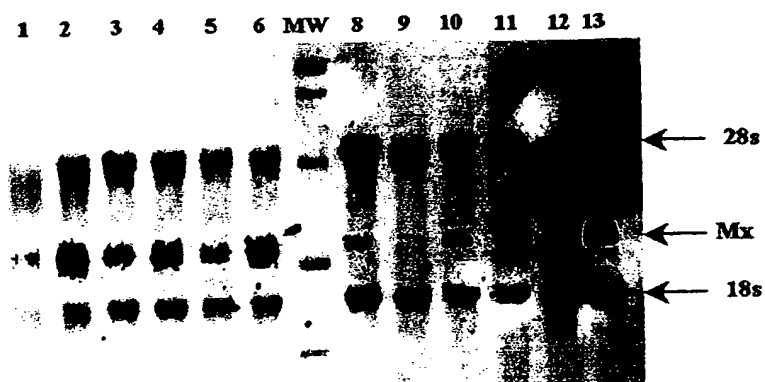


Figure 1

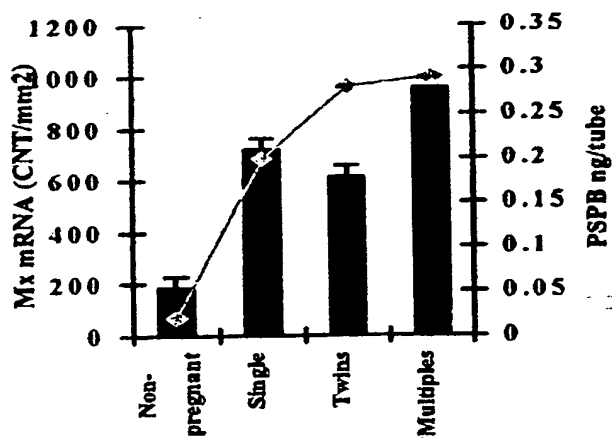


Figure 2



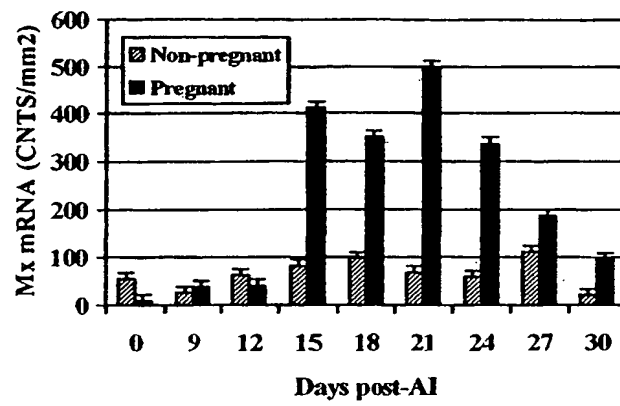


Figure 3

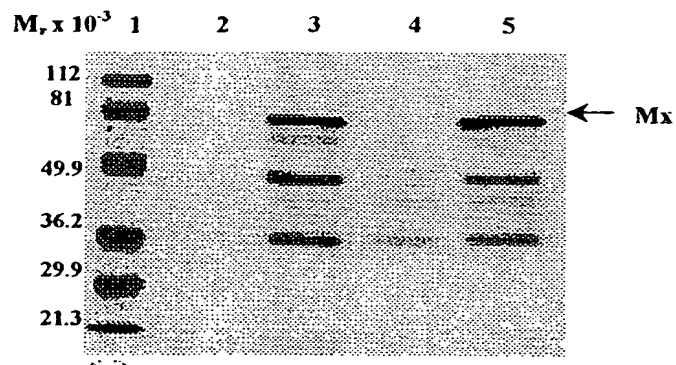


Figure 4

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/18479

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/48, 33/53

US CL : 436/65, 510

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
U.S. : 436/65, 510

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HANSEN et al, Transient Ubiquitin Cross-Reactive Protein Gene Expression in the Bovine Endometrium, Endocrinology, Vol. 138, No. 11, 1997. see entire document.	1, 2, 4, 6-9 and 11
A	JOHNSON et al, Pregnancy and Interferon-Tau Induce Conjugation of Bovine Ubiquitin Cross-Reactive Protein to Cytosolic Uterine Proteins, Biology of Reproduction, Vol. 58, 898-904 (1998), see entire document.	1-40
A	US 4,705,748 (SASSER et al) 10 November 1987 (10.11.1987), see entire document.	1-40

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

21 August 2002 (21.08.2002)

Date of mailing of the international search report

19 SEP 2002

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